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thank you,

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A Monoclonal Antibody against the c-*erbB*-2 Protein Enhances the Cytotoxicity of *cis*-Diamminedichloroplatinum against Human Breast and Ovarian Tumor Cell Lines

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ABSTRACT

A monoclonal antibody (TAb 250) specific to an extracellular epitope of the c-erbB-2 protein (gp185) inhibited the *in vitro* proliferation of human breast tumor cell lines that overexpress c-*erbB*-2 in a dose-dependent manner. Treatment of cells with combinations of *cis*-diamminedichloroplatinum (CDDP) and TAb 250 resulted in a significantly enhanced cytotoxic effect. This synergistic cytotoxicity was apparent over a wide range of antibody concentrations (200 pg/ml–100 μg/ml) including concentrations that showed no inhibitory effect alone. TAb 250 did not increase the cytotoxic effect of CDDP in a cell line exhibiting no detectable level of gp185. Athymic mice bearing s.c. xenografts of human tumor cells expressing high levels of gp185 showed a greatly enhanced inhibition of tumor growth when treated with TAb 250 and CDDP compared to treatment with the antibody or CDDP alone. This effect was specific inasmuch as TAb 250 did not enhance the growth-inhibitory effect of CDDP on tumor xenografts which were not expressing gp185.

INTRODUCTION

The c-*erbB*-2 (Her-2/*neu*) protooncogene encodes a M_r 185,000 transmembrane glycoprotein with extensive homology to the EGF[3] receptor. Studies with NIH3T3 cells have suggested a direct role for overexpression of c-*erbB*-2 in neoplastic transformation (1, 2). Amplification of the c-*erbB*-2 gene has been described in a number of cancers including human mammary and ovarian carcinomas (3–7), as well as salivary gland adenocarcinomas (8), gastric tumors, and colon adenocarcinomas (9). A survey of 189 primary breast adenocarcinomas by Slamon *et al.* (10) found that the c-*erbB*-2 gene was amplified in about 30% of the tumors and amplification was correlated with a poor disease prognosis. Immunohistochemical studies of gp185 abundance in normal human tissues show reactivity in proximal kidney tubules, mucosal epithelium in the gastrointestinal tract, and squamous epithelium in skin (6, 11–13). Most other adult tissues show little or no reactivity with antibodies against gp185 including normal breast, ovary, spleen, liver, bone marrow, prostate, adrenal, and lung (6), suggesting that this protein may be a useful therapeutic target in tumors derived from tissues where the protein is overexpressed.

Inhibition of the transformed phenotype as well as proliferation of tumor cells *in vitro* and *in vivo* by monoclonal antibodies reactive with gp185 has been reported previously. Drebin *et al.* (14) described a murine monoclonal IgG2a antibody reactive with domains of gp185 expressed on the surface of NIH3T3 cells transformed with the *neu* gene. This antibody inhibited anchorage-independent growth of these cells and significantly reduced the rate at which the cells formed tumors in nude mice. Both inhibition of colony formation in soft agar and inhibition of tumor growth in nude mice required the continuous presence of antibody, suggesting that its effects were cytostatic rather than cytotoxic. Another cytostatic monoclonal antibody reactive with gp185 has been described by Hudziak *et al.* (15). This antibody (4D5) recognizes a carbohydrate epitope on the extracellular domain of gp185, and it reversibly inhibits *in vitro* proliferation of several human breast tumor cell lines that overexpress the c-*erbB*-2 protein.

To be of significant therapeutic value, monoclonal antibodies specific for gp185, such as those described, must effectively mediate cytotoxicity either through activation of complement or cytotoxic spleen cells. An alternate approach is to mediate the efficacy of chemotherapeutic drugs. In the present study, we have investigated the effects of an anti-c-*erbB*-2 monoclonal antibody, alone and in combination with CDDP, an alkylating agent commonly used in the treatment of human neoplasms (for review, see Refs. 16 and 17). We discuss the effects of combined treatment on the proliferation of human tumor cell lines that overexpress the c-erbB-2 protein.

MATERIALS AND METHODS

Cell Culture. Human tumor cell lines, HBL100 and MDA-MB-468, were obtained from the American Type Culture Collection (Rockville, MD). SKBR-3 cells were kindly provided by Dr. S. Aaronson (NIH, Bethesda, MD), and SKOV-3 cells were a gift from Dr. D. Slamon (University of California, Los Angeles, CA).

HBL100, MDA-MB-468, and SKBR-3 cells were maintained in minimal essential medium with Earle's salts (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), and 2 mM L-glutamine. MDA-MB-468 were also supplemented with nonessential amino acids and sodium pyruvate. SKOV-3 cells were maintained in Iscove's modified Dulbecco's medium (Gibco), 10% fetal bovine serum, and 2 mM L-glutamine.

Monoclonal Antibody Preparation and Characterization. A murine monoclonal antibody, TAb 250, was prepared as described previously (18) using intact NIH3T3 cells transformed with the c-*erbB*-2 oncogene (NIH3T3$_h$, kindly provided by Dr. S. Aaronson). The antibody was screened for positive reactivity by enzyme-linked immunosorbent assay against fixed NIH3T3$_h$ and lack of reactivity against fixed nontransformed control NIH3T3 cells. Furthermore, it was also screened using a fluorescence-activated cell sorter for specific reactivity with live NIH3T3$_h$ cells. After several rounds of cloning, the hybridoma was injected into mice for ascites production. Monoclonal antibody was purified from ascites fluid by high performance liquid chromatography, dialyzed against PBS, and stored at −20°C.

Radiolabeling and Immunoprecipitation of gp185. Human tumor cell lines were cultured in T150 flasks and labeled with 400 μCi of [^{35}S] cysteine in 15 ml of cysteine-free medium (Dulbecco's modified Eagle's medium with 4.5 g/liter of glucose). Cells were labeled overnight at 37°C. Labeling medium was removed and the cells washed twice with PBS. Cells were lysed in 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 10 mg/ml bovine serum albumin, and 0.2 mM phenylmethylsulfonyl fluoride buffer and centrifuged at 100,000 × *g* for 30 min to remove insoluble material.

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[3] The abbreviations used are: EGF, epidermal growth factor; CDDP, *cis*-diamminedichloroplatinum; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide.

Prior to immunoprecipitation, supernatants were stripped of nonspecific protein A binding by incubation at 4°C for 4 h with 100 μl of a 50% slurry of protein A-Sepharose beads. The beads and nonspecifically bound material were removed by a 30-s spin in a microfuge, and supernatants were removed to new tubes. TAb 250 (20 μl containing approximately 10 μg) or anti-EGF receptor (Amersham) was then added, and the mixtures were incubated for 24 h at 4°C on a rotator. The following day, 50 μl of the protein A slurry were added to the sample which was incubated for 4 h at 4°C on a rotator. The beads were then pelleted for 30 s in a microfuge and washed five times with ice cold 100 mM Tris-HCl (pH 7.5),100 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 10 mg/ml bovine serum albumin, and 0.2 mM phenylmethylsulfonyl fluoride buffer. Between the 3rd and 4th wash, tubes were changed. The final pellet was suspended in 50 μl of Laemmli sample buffer containing 1% β-mercaptoethanol. Samples were heated to 75°C for 5 min and spun for 30 s in a microfuge, and the supernatants were electrophoresed on a 7% sodium dodecyl sulfate-polyacrylamide gel.

Following electrophoresis, the gel was fixed in 10% acetic acid-30% methanol for 1 h. After a washing in distilled water, gels were soaked for 1 h in 250 ml fresh distilled water. Gels were permeated with 250 ml EnHance (DuPont) for 90 min and equilibrated in 2% glycerol prior to drying onto filter paper. Dried gels were exposed to Kodak X-OMAT AR-5 X-ray film at −80°C for 3 days.

MTT Assay. MTT assays were carried out according to a modification of Mosmann (19). Cells were removed from tissue culture flasks with Versene 1:5000 (Gibco), centrifuged in tissue culture medium at 500 × *g* for 5 min, and resuspended in medium at a concentration of 1×10^5 cells/ml. Cells were plated (100 μl/well) into 96-well microtiter plates (Falcon) and incubated in a humidified CO_2 incubator at 37°C for 24 h.

On the next day, antibody and/or CDDP (Platinol; Bristol Myers) were added. Immediately after deposition of the highest antibody concentration into the first column of wells, 1:2 dilutions of TAb 250, or an IgG1 isotype control (Chemicon), were performed directly in the microtiter plates using a multichannel pipet. CDDP was initially diluted in normal saline at room temperature and was added to appropriate wells at concentrations indicated in the figure legends. Plates were then incubated for 3 days, followed by the addition of 10 μl/well of MTT (Sigma). MTT was prepared as a 5-mg/ml solution in PBS, filter sterilized, and stored at 4°C in the dark. Plates were kept dark and incubated for an additional 4 h at 37°C. The MTT crystals were dissolved by mixing the contents of the wells vigorously with 100 μl of isopropanol containing 0.04 N HCl and 3% sodium dodecyl sulfate. Absorbance at 570 nm was determined using an enzyme-linked immunosorbent assay reader.

In Vivo Subcutaneous Xenografts. Female BALb/c-*nu*/*nu* mice (6–8 weeks old) were implanted s.c. with 5×10^6-1×10^7 SKOV-3 or MDA-MB-468 cells. Tumors were measured every 3–4 days with vernier calipers and tumor volume was calculated as the product of length x width x height. Animals were treated (6–8 animals per group) via tail vein or i.p. injection every 7 days for 3 treatment cycles. Mice received either an isotype-matched IgG1 antibody, TAb 250, CDDP, or a combination treatment. Mice receiving combination treatments were given injections of CDDP 45 min after injection of antibody. Statistical significance was determined by an analysis of the natural logarithms of the tumor volumes. Zero tumor volumes were set equal to 1 to permit use of the log transformation. For each mouse, the method of least squares was used to fit a straight line to the log tumor volumes as a function of time. Analysis of variance models were fit to the estimated slopes, and the Tukey multiple comparison approach was used to test for between-treatment group differences (20). An overall experiment-wise significance level of $\alpha = 0.05$ was used for the pairwise comparisons.

RESULTS

To determine the reactivity and specificity of TAb 250 for gp185 in human tumor cell lines, radiolabeled whole cell lysates were incubated and precipitated with TAb 250, a nonspecific IgG1 isotype control antibody, and murine monoclonal antibody reactive with the EGF receptor. As shown in Fig. 1, TAb 250 precipitates a protein with a molecular weight of 185,000 from NIH3T3, SKBR-3, and SKOV-3 cells, whereas the isotype control antibody shows no reactivity (data not shown). Fig. 1 also shows that an EGF receptor antibody precipitates a distinct and separate M_r 170,000 protein that is particularly abundant in MDA-MB-468 cells, which are known to overexpress the EGF receptor (21).

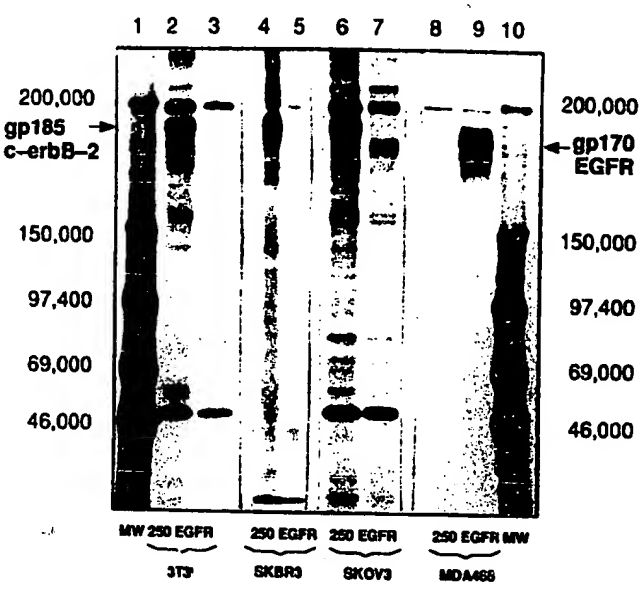

Fig. 1. Specificity of TAb 250. 3T3, (*Lanes 2–3*), SKBR-3 (*Lanes 4–5*), SKOV-3 (*Lanes 6–7*), and MDA-MB-468 (*Lanes 8–9*) cells were labeled with [^{35}S] cysteine and cell lysates were immunoprecipitated with TAb 250 (*Lanes 2, 4, 6, 8*) or an anti-EGF receptor antibody (*EGFR*; *Lanes 3, 5, 7, 9*) as described in "Materials and Methods." Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. *Lanes 1* and *10*, molecular weight (*MW*) markers.

The antiproliferative effects of TAb 250 *in vitro* were tested on cell lines using a 72-h colorimetric MTT assay. Growth of SKBR-3 cells, a human metastatic breast tumor line that expresses high levels of gp185, was inhibited 40–50% of either untreated cells or cells exposed to a nonspecific isotype control antibody (Fig. 2). This effect decreased with dilution of the antibody such that there was no significant difference in proliferation between cells treated with 0.8 μg/ml TAb 250 or the IgG1 isotype control. MDA-MB-468 cells, which express high numbers of EGF receptors (21), but an undetectable level of c-erbB-2 protein (as determined by ^{125}I-TAb 250 binding[4]), or the immortalized breast cell line, HBL100, were not affected by treatment with TAB 250. These data suggest that TAb 250 could specifically inhibit *in vitro* proliferation of cells expressing high levels of c-*erbB*-2, and that cells lacking this protein or expressing high levels of EGF receptor were unaffected.

The nature of the growth inhibition of SKBR-3 cells was further investigated and the antibody effects were found to be cytostatic. Cells resumed proliferation following a 72-h incubation with antibody if the cells were washed, refed with culture medium lacking TAb 250, and incubated for an additional 5 days (data not shown).

Because a cytostatic antibody would not be likely to provide significant antitumor efficacy, we combined TAb 250 with

[4] L. K. Shawver *et al.*, unpublished observation.

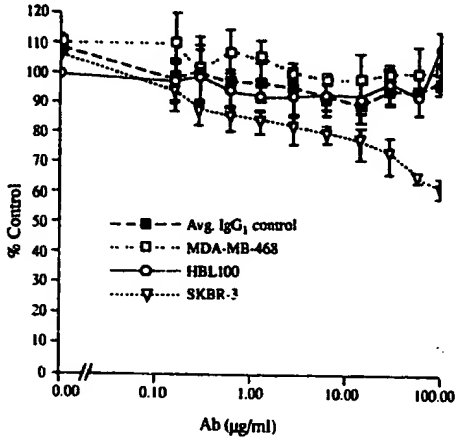

Fig. 2. Inhibitory effects of TAb 250 on human breast cell lines. SKBR-3, MDA-MB-468, and HBL100 were obtained from the American Type Culture Collection and grown to confluence in minimum essential medium containing 10% fetal bovine serum and L-glutamine. Growth inhibition was determined using a MTT assay as described in "Materials and Methods." *Ab*, antibody.

chemotherapeutic drugs and evaluated proliferation of tumor cells *in vitro* and *in vivo*. Fig. 3*A* shows that cells in culture exposed simultaneously to TAb 250 and CDDP were dramatically inhibited. During the 72-h incubation period, cells exposed to 1.0 or 2 μg/ml CDDP reduced proliferation to 70 and 55% of control. However, cells treated with 1.0 μg/ml CDDP plus TAb 250 were inhibited to 25–30% of control, and those treated with 2.0 μg/ml CDDP and TAb 250 were inhibited to 10–15% of control. Treatment of cells with CDDP and an isotype control antibody did not inhibit proliferation greater than treatment with CDDP alone (data not shown).

The combined effect of TAb 250 and CDDP is specific for cells expressing c-*erbB*-2 as shown in Fig. 3*B*. The growth of MD-MB-468 cells was not affected by TAb 250 alone, even at high concentrations. In addition, the inhibitory effect of CDDP, when combined with TAb 250, was not greater than CDDP alone. While MDA-MB-468 cells appear to be more sensitive to CDDP, this may reflect variation between cell lines rather than expression of c-*erbB*-2 or the EGF receptor. Regardless of the difference in CDDP effect, a greater sensitivity was not observed by treatment of the MDA-MB-468 cells with TAb 250.

When SKBR-3 cells were treated with antibody and CDDP, followed by incubation with fresh growth medium for an additional 5 days, no evidence of cell growth was observed suggesting the combination was cytotoxic (Fig. 4). This synergistic cytotoxicity was apparent over a wide range of antibody concentrations (200 pg/ml–100 μg/ml; data not shown) and could be observed even at antibody concentrations that did not appear to have an effect when used alone (see Figs. 3*A* and 4). In addition, the synergistic effect was most readily observed when the dose of CDDP used alone resulted in a 30–50% inhibition. Under these conditions, the combined treatment resulted in 80–100% cytotoxicity. Time course experiments (data not shown) suggested that the effects of the combined treatment occurred within the first 24 h of antibody and drug exposure.

Because of the marked effect observed *in vitro*, the combination of CDDP and TAb 250 was assayed for inhibition of growth of s.c. xenografts in athymic mice (Fig. 5). Seven days following tumor inoculation, animals were treated with TAb 250, IgG1, or the combination of TAb 250 and CDDP. Treatment of tumor bearing animals with CDDP alone significantly inhibited tumor growth ($P < 0.05$). Treatment with 500 μg TAb 250 alone did not significantly inhibit tumor growth ($P > 0.05$). However, the inhibitory effect on tumor growth was greatly enhanced by injecting 500 μg of TAb 250 followed by treatment with CDDP as observed in Fig. 5*A*. The combined treatment was significantly better than either of the treatments alone ($P < 0.05$) suggesting that these agents are acting synergistically. The weight of the animals did not change during the course of treatment and no other toxicities were observed. Tumors from animals treated with CDDP and TAb 250 resumed growth after day 42 although at a much reduced rate (data not shown). The *in vivo* effect of CDDP combined with TAb 250 appears to be specific for cells expressing c-*erbB*-2 because the combination effect on animals bearing tumors from MDA-MB-468 cells was not significantly greater than the inhibition observed with CDDP alone (Fig. 5*B*).

The combination effects of TAb 250 and CDDP were significantly challenged by examining their effect on a tumor burden that was 6-fold greater at the beginning of treatment than described for Fig. 5. Fig. 6 shows that treatment of tumors with established growth are significantly inhibited with CDDP or with TAb 250 ($P < 0.05$). However, while the combined treatment resulted in a >65% reduction of tumor growth, this was not significantly greater than treatment with TAb 250 or CDDP alone.

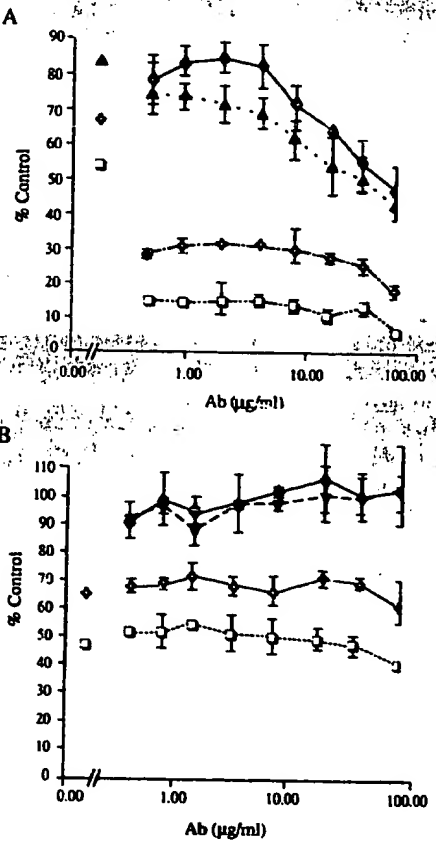

Fig. 3. Synergistic effect of TAb 250 and CDDP. SKBR-3 cells (*A*) or MDA-MB-468 cells (*B*) were cultured and growth inhibition by TAb 250 and CDDP is carried out using a MTT assay as described in "Materials and Methods." For combination treatment, antibody was added to the wells at the concentrations indicated followed by addition of CDDP. *A*. —◆—, TAb 250; ▲, CDDP (0.5 μg/ml); ◆, CDDP, (1 μg/ml), □, CDDP, (2 μg/ml); --▲--, TAb 250 plus CDDP (0.5 μg/ml); —◆—, TAb 250 plus CDDP (1 μg/ml); —□—, TAb 250 plus CDDP (2 μg/ml). *B*, --▼--, IgG_1; —◆—, TAb 250; ◆, CDDP (0.1 μg/ml); □, CDDP (0.2 μg/ml); ·····◆·····, TAb 250 plus CDDP (0.1 μg/ml); ···□···, TAb 250 plus CDDP (0.2 μg/ml).

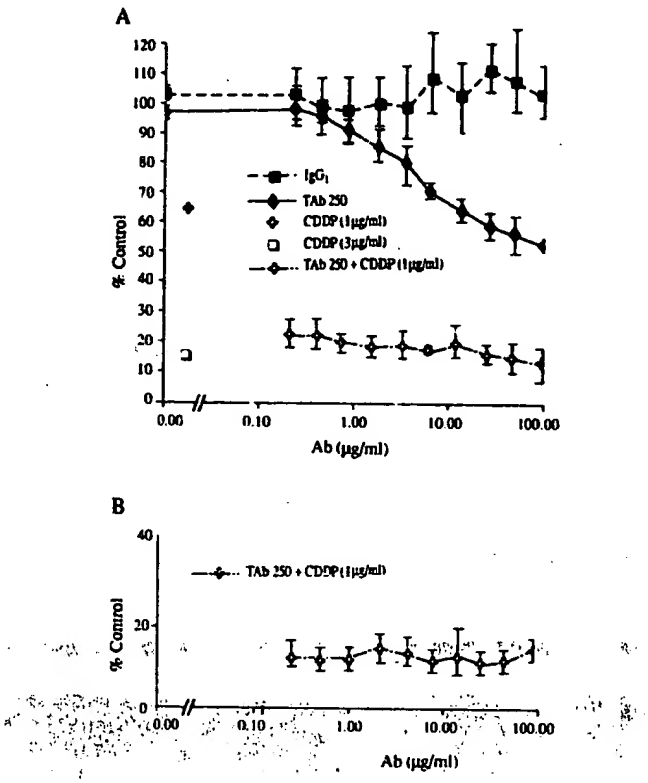

Fig. 4. Cytotoxic effects of TAb 250 and CDDP. In *A*, SKBR-3 cells were cultured, and growth inhibition by TAb 250 and CDDP was carried out using a MTT assay as described in "Materials and Methods." In *B*, following 3 days exposure to TAb 250 and CDDP, the cells were gently washed, refed growth medium, and incubated for an additional 5 days prior to addition of MTT.

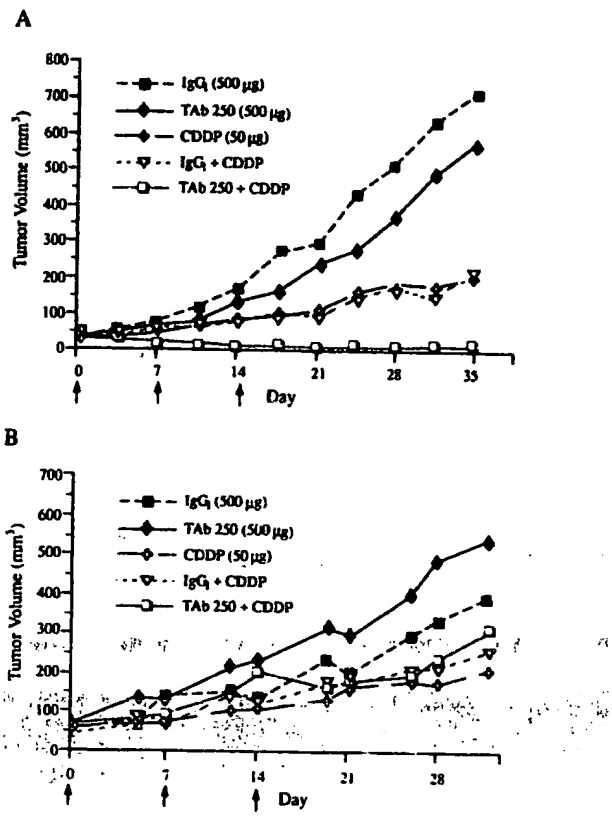

Fig. 5. Antitumor activity of TAb 250 in combination with CDDP. In *A*, SKOV-3 cells (1×10^7) were implanted s.c. into athymic mice and allowed to grow until reaching a volume of 25–40 mm³. Three injections of TAb 250, IgG1, CDDP, or TAb 250 followed by CDDP 45 min later were administered once a week for 3 weeks (*arrows*). Tumor parameters were measured twice a week with a caliper and tumor volume calculated as

$$\text{Tumor volume (mm}^3\text{)} = \text{length} \times \text{width} \times \text{height}$$

In *B*, MDA-MB-468 cells were implanted into athymic mice as described in *A*. Following inoculation, tumors were allowed to grow to a volume of 50–100 mm³ prior to treatment. Animals were treated as described in *A*.

DISCUSSION

Several antibodies against the c-erbB-2 protein have been shown to inhibit the growth of cell lines overexpressing c-*erbB*-2. Hudziak *et al.* (15) demonstrated antiproliferative effects of a c-erbB-2 monoclonal antibody against human tumor cell lines *in vitro*, and Drebin *et al.* (14) showed that growth of 3T3 cells transformed with *neu* could be inhibited in soft agar and in nude mice with a monoclonal antibody made against the rat neu protein. The effects demonstrated in these studies, however, were reversible or required the continuous presence of antibody. Thus, to be of significant therapeutic value, monoclonal antibodies to gp185 are likely to require conjugation or combination with other cytotoxic agents. Recently, an antibody against the EGF receptor was reported to have enhanced antitumor activity *in vivo* when combined with CDDP (22). In the present study, we show that combining CDDP with a monoclonal antibody specific for the extracellular domain of c-*erbB*-2 markedly enhances the inhibitory effect of CDDP both *in vitro* and *in vivo*.

SKBR-3 cells exposed to TAb 250 and CDDP were dramatically inhibited compared to cells exposed to either TAb 250 or CDDP alone. While the inhibitory effect of cells exposed to TAb 250 alone was cytostatic, the inhibitory effect appeared to be cytotoxic for cells exposed to both antibody and drug. The synergistic inhibition was apparent f r antibody concentrati ns which did not appear to have an effect when used alone.

TAb 250 also markedly enhanced the inhibitory effect of CDDP *in vivo* using a s.c. xenograft model with SKOV-3 cells. This increased inhibitory effect was most readily observed when treatment began early after tumor cell inoculation. The effect

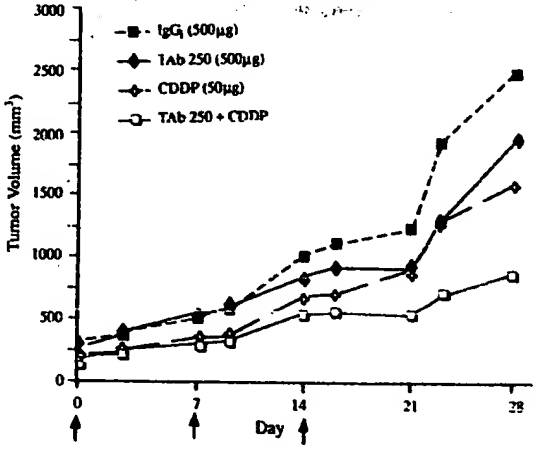

Fig. 6. Inhibitory effects of TAb 250 and CDDP on established tumor growth. SKOV-3 cells were implanted into athymic mice as described for Fig. 5. However, following inoculation, tumors were allowed to grow to a volume of 150–200 mm³ prior to treatment. Animals were then treated once a week for 3 weeks with TAb 250, IgG1, CDDP, or combination as described for Fig. 5.

was less significant when treating animals with established tumor growth. However, increasing the frequency of dosing with antibody or increasing the amount of antibody injected for each dose may result in increased efficacy and we are currently examining these parameters.

The mechanism for the synergistic effect of TAb 250 and CDDP remains unclear at this time. While CDDP has significant effects on DNA alkylation, its therapeutic effects on human cancers may occur due to several mechanisms. K562 and L1210 cells treated with CDDP have been shown to have decreased methionine uptake and altered endogenous folate and methionine metabolism (23, 24). Several components of the growth factor-induced signal transduction pathway are affected by CDDP. Inhibitors of protein kinase C have been shown to enhance the antiproliferative activity of CDDP (25, 26). In addition, gene expression of c-*fos* has been shown to increase in Chinese hamster ovary cells treated with CDDP (27). Both c-*fos* and c-*ras* have been demonstrated to be amplified in patients failing treatment with CDDP (28). Since TAb 250 is directed against the extracellular domain of the c-erbB-2 protein and may possibly interfere with ligand binding, the synergistic effect with CDDP may be due to an interaction along this common pathway.

An alternative explanation for a synergistic enhancement may be due to an inhibition of DNA repair. Treatment of cells with inhibitors of poly(ADP-ribose) polymerase has been documented to depress the excision repair of alkylated DNA (29–31). A similar phenomenon may occur after treatment with CDDP and TAb 250. The effects of TAb 250 on the repair of DNA-interstrand cross-links is being investigated.

The c-*erbB*-2 oncogene is amplified and overexpressed in a large number of cell lines derived from human adenocarcinomas. Of significance is the percentage of primary adenocarcinomas of the breast which overexpress the c-erbB-2 protein (10) and the correlation with a poor disease prognosis. The extracellular portion of the c-erbB-2 protein provides an attractive target for immunotherapeutic modalities. However, treatment using antibodies alone may be reversible or may require the continued presence of antibody. The use of monoclonal antibodies as adjunctive therapy with CDDP provides an alternative means of therapy for human tumors which overexpress c-*erbB*-2.

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